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(54) Title: METHODS OF TREATMENT USING DUAL MATRIX-METALLOPROTEINASE-2 AND MATRIX METALLOPROTEINASE-9 INHIBITORS

(57) Abstract: The invention relates to a method for treating a patient suffering from pain or stroke, said method comprising the step of administering to the patient a pain-treating effective amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier.

METHODS OF TREATMENT USING DUAL MATRIX-METALLOPROTEINASE-2 AND MATRIX METALLOPROTEINASE-9 INHIBITORS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims benefit to the earlier provisional U.S. Application No. 60/158,787, filed on October 12, 1999, the contents of which are incorporated herein by reference in their entirety.

FIELD OF INVENTION

This invention relates to methods for treating pain in a patient, comprising administering to the patient a pain-reducing effective amount of a dual inhibitor of matrix metalloproteinase-2 (herein "MMP-2") and matrix metalloproteinase-9 (herein "MMP-9").

BACKGROUND OF THE INVENTION

The extracellular matrix (ECM) is a multifunctional complex of proteins and proteoglycans assembled in a highly organized manner that contributes to the structural integrity of cells and tissue within an organ system. The basement membrane, which provides structural support to the vasculature, is comprised of ECM molecules such as type IV collagen, laminin, and fibronectin. Various factors are involved in maintaining the integrity of the ECM and the tissues it supports. However, in certain pathological circumstances, the ECM is modulated such that the structure of the tissue becomes damaged or destroyed. The matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes that degrade the molecules of the extracellular matrix. Two members of the MMP family, MMP-2 (72 kDa gelatinase/Gelatinase A) and MMP-9 (92 kDa gelatinase/Gelatinase B), degrade the ECM components of the basement membrane. Their substrates include types IV and V collagen, fibronectin, elastin, and denatured interstitial collagens. Matrix degradation attributed to these proteinases has been shown to play an

important role in the progression of diseases such as atherosclerosis, inflammation, stroke, and tumor growth and metastasis.

Nerve injury caused by constriction results in ischemia of the nerve tissue and, ultimately, neuronal cell death. Nerve injury following constriction is primarily a result of the decrease in blood flow and of energy depletion due to compression of microvessels which supply the nervous tissue. These events cause the nerve tissue to become infarcted, with contributions from excitotoxicity, enzyme activation, edema, and inflammation. A significant inflammatory response occurs following nerve injury. For example, neutrophils infiltrate the damaged tissue and contribute to the nerve injury, further exacerbating the injury response. Further, researchers have demonstrated that neutrophils utilize MMPs for their migration. It is believed that MMP inhibition would prevent or ameliorate the tissue damage that occurs following nerve injury. Further MMP inhibition would prevent or reduce the degree of inflammatory cell infiltration into the damaged tissue.

Clearly, there is a need for identification and characterization of dual antagonists of MMP-2 and MMP-9 that play a role in preventing, ameliorating or correcting stroke; hemorrhage; reperfusion injury; cerebral ischemia; cerebral infarction; enhanced or exaggerated sensitivity to pain, such as hyperalgesia, causalgia and allodynia; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional pain syndromes I and II; arthritic pain; sports injury pain; pain related to viral infection, *e.g.,* HIV, post-polio syndrome, and post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; post-chemotherapy pain; post-stroke pain; post-operative pain; physiological pain; inflammatory pain; acute inflammatory conditions/visceral pain, *e.g.,* angina, irritable bowel syndrome (IBS), and inflammatory bowel disease; neuropathic pain; neuralgia; painful diabetic neuropathy; traumatic nerve injury; spinal cord injury; and tolerance to narcotics or withdrawal from narcotics, among others.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a method for treating pain in a patient, said method comprising the step of administering to the patient a pain-treating effective amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier, wherein the patient is suffering from enhanced or exaggerated sensitivity to pain, such as hyperalgesia, causalgia and allodynia; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional pain syndromes I and II; arthritic pain; sports injury pain; pain related to viral infection, *e.g.*, HIV, post-polio syndrome, and post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; post-chemotherapy pain; post-stroke pain; post-operative pain; physiological pain; inflammatory pain; acute inflammatory conditions/visceral pain, *e.g.,* angina, irritable bowel syndrome (IBS), and inflammatory bowel disease; neuropathic pain; neuralgia; painful diabetic neuropathy; traumatic nerve injury; spinal cord injury; and tolerance to narcotics or withdrawal from narcotics.

In a second aspect, the invention relates to a method for treating nerve tissue damage in a patient in need thereof, said method comprising the step of administering an effective nerve tissue damage-reducing amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier, wherein the patient is suffering from stroke; hemorrhage; reperfusion injury; cerebral ischemia; cerebral infarction; enhanced or exaggerated sensitivity to pain, such as hyperalgesia, causalgia and allodynia; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional pain syndromes I and II; arthritic pain; sports injury pain; pain related to viral infection, *e.g.*, HIV, post-polio syndrome, and post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; post-chemotherapy pain; post-stroke pain; post-operative pain; physiological pain; inflammatory pain; acute inflammatory conditions/visceral pain, *e.g.,* angina, irritable bowel syndrome (IBS), and inflammatory bowel disease; neuropathic pain; neuralgia; painful diabetic neuropathy; traumatic nerve injury; spinal cord injury; and tolerance to narcotics or withdrawal from narcotics.

In a third aspect, the invention relates to a method for treating a patient suffering from a disease selected from the group consisting of: stroke, hemorrhage, reperfusion injury, cerebral ischemia,, and cerebral infarction, said method comprising the step of administering an effective amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence from human MMP-2 (SEQ ID NO:1).

Figure 2 shows the deduced amino acid sequence from human MMP-2 (SEQ ID NO:2).

Figure 1 shows the nucleotide sequence from human MMP-9 (SEQ ID NO:3).

Figure 2 shows the deduced amino acid sequence from human MMP-9 (SEQ ID NO:4).

DESCRIPTION OF THE INVENTION

Definitions

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"MMP-2" refers, among others, generally to a polypeptide having the amino acid sequence set forth in SEQ ID NO:2 or an allelic variant thereof.

"MMP-9" refers, among others, generally to a polypeptide having the amino acid sequence set forth in SEQ ID NO:4 or an allelic variant thereof.

"MMP-2 activity" or "MMP-2 polypeptide activity" or "biological activity of the MMP-2 polypeptide" refers to the metabolic or physiologic function of human MMP-2, including similar activities or improved activities or these activities with decreased undesirable side-effects.

"MMP-9 activity" or "MMP-9 polypeptide activity" or "biological activity of MMP-9 or MMP-9 polypeptide" refers to the metabolic or physiologic function of

human MMP-9, including similar activities or improved activities or these activities with decreased undesirable side-effects.

"MMP-2 gene" refers to a polynucleotide having the nucleotide sequence set forth in SEQ ID NO:1 or allelic variants thereof and/or their complements.

"MMP-9 gene" refers to a polynucleotide having the nucleotide sequence set forth in SEQ ID NO:3 or allelic variants thereof and/or their complements.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library.

"Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA

characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques that are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Co, New York, 1993 and Wold, F., Posttranslational Protein Modifications:

Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter, *et al.*, *Meth. Enzymol.* (1990) 182: 626-646 and Rattan, *et al.*, *Ann NY Acad Sci* (1992) 663:48-62.

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

"Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, 1988; BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, 1993;

COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., *SIAM J. Applied Math.*, 48: 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., *et al., Nucleic Acids Research* 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, *et al., J. Molec. Biol.* 215: 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, *et al.,* NCBI NLM NIH Bethesda, MD 20894; Altschul, S., *et al., J. Mol. Biol.* 215: 403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following:

1) Algorithm: Needleman, *et al., J. Mol Biol.* 48: 443-453 (1970)

Comparison matrix: BLOSSUM62 from Hentikoff, *et al., Proc. Natl. Acad. Sci. USA.* 89:10915-10919 (1992).

Gap Penalty: 12

Gap Length Penalty: 4

A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

Preferred parameters for polynucleotide comparison include the following:

1) Algorithm: Needleman, *et al., J. Mol Biol.* 48: 443-453 (1970).

Comparison matrix: matches = +10, mismatch = 0

Gap Penalty: 50

Gap Length Penalty: 3

Available as: The "gap" program from Genetics Computer Group, Madison WI. These are the default parameters for nucleic acid comparisons.

By way of example, a polynucleotide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:1, that is 100% identical, or it may include up to a certain integer number of nucleotide alterations as compared to the reference sequence. Such alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The number of nucleotide alterations is determined by multiplying the total number of nucleotides in SEQ ID NO:1 by the numerical percent of the respective percent identity(divided by 100) and subtracting that product from said total number of nucleotides in SEQ ID NO:1, or:

$$n_n \leq x_n - (x_n \bullet y),$$

wherein n_n is the number of nucleotide alterations, x_n is the total number of nucleotides in SEQ ID NO:1, and y is, for instance, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%, *etc.*, and wherein any non-integer product of x_n and y is rounded down to the nearest integer prior to subtracting it from x_n. Alterations of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 may create nonsense, missense or frameshift mutations in this coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

Similarly, a polypeptide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:2, that is 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the % identity is less than 100%. Such alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference

polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of amino acid alterations for a given % identity is determined by multiplying the total number of amino acids in SEQ ID NO:2 by the numerical percent of the respective percent identity(divided by 100) and then subtracting that product from said total number of amino acids in SEQ ID NO:2, or:

$$n_a \leq x_a - (x_a \bullet y),$$

wherein n_a is the number of amino acid alterations, x_a is the total number of amino acids in SEQ ID NO:2, and y is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, *etc.*, and wherein any non-integer product of x_a and y is rounded down to the nearest integer prior to subtracting it from x_a.

Polypeptides of the Invention

In one aspect, the present invention relates to MMP-2 polypeptides. The MMP-2 polypeptides include the polypeptide of SEQ ID NO:2; as well as polypeptides comprising the amino acid sequence of SEQ ID NO:2; and polypeptides comprising an amino acid sequence having at least a 95% identity to that of SEQ ID NO:2 over its entire length. Preferably, MMP-2 polypeptides exhibit at least one biological activity of human MMP-2.

In another aspect, the present invention relates to MMP-9 polypeptides. The MMP-9 polypeptides include the polypeptide of SEQ ID NO:4; as well as polypeptides comprising the amino acid sequence of SEQ ID NO:4; and polypeptides comprising an amino acid sequence having at least a 95% identity to that of SEQ ID NO:4 over its entire length. Preferably, MMP-9 polypeptides exhibit at least one biological activity of human MMP-9.

The MMP-2 and MMP-9 polypeptides may be in the form of the "mature" protein or may be a part of a larger protein such as a fusion protein. It is often advantageous to include an additional amino acid sequence that contains secretory or leader sequences, pro-sequences, sequences that aid in purification such as multiple

histidine residues, or an additional sequence for stability during recombinant production.

Biologically active fragments of the MMP-2 and MMP-9 polypeptides are also included in the invention. A fragment is a polypeptide having an amino acid sequence that entirely is the same as part, but not all, of the amino acid sequence of the aforementioned MMP-2 and MMP-9 polypeptides. As with MMP-2 and MMP-9 polypeptides, fragments may be "free-standing," or comprised within a larger polypeptide of which they form a part or region, most preferably, as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments from about amino acid number 1-20, 21-40, 41-60, 61-80, 81-100, and 101 to the end of the human MMP-2 and MMP-9 polypeptides. In this context, "about" includes the particularly recited ranges larger or smaller by several, 5, 4, 3, 2 or 1 amino acid at either extreme or at both extremes.

Preferred fragments include, for example, truncation polypeptides having the amino acid sequence of MMP-2 and MMP-9 polypeptides, except for deletion of a continuous series of residues that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus or deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Also preferred are fragments characterized by structural or functional attributes such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Biologically active fragments are those that mediate MMP-2 or MMP-9 activity, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Also included are those fragments that are antigenic or immunogenic in an animal, especially in a human.

Preferably, all of these polypeptide fragments retain the biological activity of MMP-2 or MMP-9, including antigenic activity. Variants of the defined sequence and fragments also form part of the present invention. Preferred variants are those that vary from the referents by conservative amino acid substitutions -- *i.e.*, those that

substitute a residue with another of like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination.

The human MMP-2 and MMP-9 polypeptides of the invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

Polynucleotides of the Invention

Another aspect of the invention relates to MMP-2 polynucleotides. MMP-2 polynucleotides include isolated polynucleotides encoding the MMP-2 polypeptides and fragments, and polynucleotides closely related thereto. More specifically, the MMP-2 polynucleotides of the invention include a polynucleotide comprising the nucleotide sequence set forth in SEQ ID NO:1 encoding a human MMP-2 polypeptide of SEQ ID NO:2, and a polynucleotide having the particular sequence of SEQ ID NO:1. MMP-2 polynucleotides further include a polynucleotide comprising a nucleotide sequence having at least a 95% identity to a nucleotide sequence encoding the human MMP-2 polypeptide of SEQ ID NO:2 over its entire length, and a polynucleotide having at least a 95% identity SEQ ID NO:1 over its entire length. In this regard, polynucleotides at least 97% identical are particularly preferred, and those with at least 98-99% are most highly preferred, with at least 99% being the most preferred. Also included under MMP-2 polynucleotides are nucleotide sequences having sufficient identity to a nucleotide sequence contained in SEQ ID NO:1 to hybridize under conditions useable for amplification or for use as a probe or marker. The invention also provides polynucleotides that are complementary to such MMP-2 polynucleotides.

Another aspect of the invention relates to MMP-9 polynucleotides. MMP-9 polynucleotides include isolated polynucleotides encoding the MMP-9 polypeptides

and fragments, and polynucleotides closely related thereto. More specifically, the MMP-9 polynucleotides of the invention include a polynucleotide comprising the nucleotide sequence set forth in SEQ ID NO:3 encoding a human MMP-9 polypeptide of SEQ ID NO:4, and a polynucleotide having the particular sequence of SEQ ID NO:3. MMP-9 polynucleotides further include a polynucleotide comprising a nucleotide sequence having at least a 95% identity to a nucleotide sequence encoding the human MMP-9 polypeptide of SEQ ID NO:4 over its entire length, and a polynucleotide having at least a 95% identity to SEQ ID NO:3 over its entire length. In this regard, polynucleotides at least 97% identical are particularly preferred, and those with at least 98-99% are most highly preferred, with at least 99% being the most preferred. Also included under MMP-9 polynucleotides are nucleotide sequences having sufficient identity to a nucleotide sequence contained in SEQ ID NO:3 to hybridize under conditions useable for amplification or for use as a probe or marker. The invention also provides polynucleotides that are complementary to such MMP-9 polynucleotides.

The polynucleotides of the present invention encoding MMP-2 and MMP-9 may be obtained using standard cloning and screening, from a cDNA library derived from mRNA in cells of human endothelial cells, peripheral blood leukocytes, spleen, thymus, brain, lung, heart, placenta, etc., using the expressed sequence tag (EST) analysis (Adams, *et al. Science* 252:1651-1656 (1991); Adams, *et al.*, *Nature*, *355*:632-634 (1992); Adams, *et al.*, *Nature* 377 Supp:3-174 (1995)). Polynucleotides of the invention can also be obtained from natural sources such as genomic DNA libraries or can be synthesized using well known and commercially available techniques.

The nucleotide sequence encoding MMP-2 polypeptide of SEQ ID NO:2 may be identical over its entire length to the coding sequence set forth in Figure 1 (SEQ ID NO:1), or may be a degenerate form of this nucleotide sequence encoding the polypeptide set forth in Figure 1 (SEQ ID NO:2), or may be highly identical to a nucleotide sequence that encodes the polypeptide of SEQ ID NO:2. Preferably, the polynucleotides of the invention comprise a nucleotide sequence that is highly identical, at least 95% identical, with a nucleotide sequence encoding a MMP-2

polypeptide, or at least 95% identical with the polynucleotide sequence contained in Figure 1 (SEQ ID NO:1) encoding MMP-2 polypeptide, or at least 95% identical to a nucleotide sequence encoding the polypeptide set forth in Figure 2 (SEQ ID NO:2).

The nucleotide sequence encoding MMP-9 polypeptide of SEQ ID NO:4 may be identical over its entire length to the coding sequence set forth in Figure 3 (SEQ ID NO:3), or may be a degenerate form of this nucleotide sequence encoding the polypeptide set forth in Figure 4 (SEQ ID NO:4), or may be highly identical to a nucleotide sequence that encodes the polypeptide of SEQ ID NO:4. Preferably, the polynucleotides of the invention comprise a nucleotide sequence that is highly identical, at least 95% identical, with a nucleotide sequence encoding MMP-9 polypeptide, or at least 95% identical with the polynucleotide sequence contained in Figure 3 (SEQ ID NO:3) encoding MMP-9 polypeptide, or at least 95% identical to a nucleotide sequence encoding the polypeptide set forth in Figure 4 (SEQ ID NO:4).

When the polynucleotides of the invention are used for the recombinant production of MMP-2 and MMP-9 polypeptide, the polynucleotide may include the coding sequence for the mature polypeptide or a fragment thereof, by itself; the coding sequence for the mature polypeptide or fragment in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions. For example, a marker sequence that facilitates purification of the fused polypeptide can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz, *et al.*, *Proc Natl Acad Sci USA* 86: 821-824 (1989), or is an HA tag. The polynucleotide may also contain non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Further preferred embodiments are polynucleotides encoding MMP-2 variants that comprise the amino acid sequence of MMP-2 polypeptide of Figure 2 (SEQ ID NO:2) in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acid residues are substituted, deleted or added, in any combination. Still further preferred embodiments are polynucleotides encoding MMP-9 variants that comprise the amino acid sequence of

MMP-9 polypeptide of Figure 4 (SEQ ID NO:4) in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acid residues are substituted, deleted or added, in any combination.

The present invention further relates to polynucleotides that hybridize to the herein above-described sequences. In this regard, the present invention especially relates to polynucleotides that hybridize under stringent conditions to the herein above-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and, preferably, at least 97% identity between the sequences.

Polynucleotides of the invention, which are identical or sufficiently identical to the nucleotide sequences contained in SEQ ID NO:1 or 3, may be used as hybridization probes for cDNA and genomic DNA, to isolate full-length cDNAs and genomic clones encoding MMP-2 or MMP-9 polypeptides and to isolate cDNA and genomic clones of other genes that have a high sequence similarity to the MMP-2 and MMP-9 genes. Such hybridization techniques are known to those of skill in the art. Typically, these nucleotide sequences are at least 95% identical to that of the referent. The probes generally will comprise at least 15 nucleotides. Preferably, such probes will have at least 30 nucleotides and may have at least 50 nucleotides. Particularly preferred probes will range between 30 and 50 nucleotides.

In one embodiment of the invention, obtaining a polynucleotide encoding MMP-2 or MMP-9 comprises the steps of screening an appropriate library under stringent hybridization conditions with a labeled probe having the nucleotide sequence of SEQ ID NO: 1 or 3 or a fragment thereof; and isolating full-length cDNA and genomic clones containing said polynucleotide sequence. Such hybridization techniques are well known to those of skill in the art. Stringent hybridization conditions are as defined above or alternatively conditions under overnight incubation at 42°C in a solution comprising: 50% formamide, 5xSSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10 % dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics to animal and human disease.

Vectors, Host Cells, Expression

The present invention also relates to vectors that comprise a polynucleotide or polynucleotides of the present invention, and host cells that are genetically engineered with vectors of the invention and to the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis, *et al.*, *BASIC METHODS IN MOLECULAR BIOLOGY* (1986) and Sambrook, *et al., MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used. Such systems include, among others, chromosomal, episomal and virus-derived systems, *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox

viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook, *et al.*, *MOLECULAR CLONING, A LABORATORY MANUAL* (*supra*).

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the desired polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

If the MMP-2 or MMP-9 polypeptide is to be expressed for use in screening assays, generally, it is preferred that the polypeptide be produced at the surface of the cell. In this event, the cells may be harvested prior to use in the screening assay. If MMP-2 or MMP-9 polypeptide is secreted into the medium, the medium can be recovered in order to recover and purify the polypeptide; if produced intracellularly, the cells must first be lysed before the polypeptide is recovered.

MMP-2 or MMP-9 polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

Screening Assays

Human MMP-2 and MMP-9 are ubiquitous in the mammalian host, and are thus responsible for many biological functions, including many pathologies.

Accordingly, it is desirous to find compounds and drugs that stimulate or inhibit the function of both human MMP-2 and MMP-9.

The *in vitro* data disclosed in Example 1 demonstrate that early increased MMP-9 expression in endothelial cells and infiltrating neutrophils is a significant response to cerebral focal ischemia, and that selective inhibition of MMP-9 activity can significantly reduce brain injury following stroke. The expression of MMP-2 also participates in tissue damage, but early expression may contribute to wound healing. These results demonstrate that a dual MMP-9/MMP-2 inhibitor would be of benefit for patients suffering from stroke, hemorrhage, reperfusion injury, cerebral ischemia, and cerebral infarction.

The *in vitro* data disclosed in Example 2 indicate that MMPs, in particular MMP-9 and MMP-2, contribute to the tissue damage and inflammation that occurs following nerve injury. These events contribute to both acute and chronic pain experienced with injury. The results in this experiment demonstrate that inhibition of MMP-9 and/or MMP-2 expression and/or activity can significantly reduce nerve tissue damage and pain following injury.

In a preferred embodiment, the invention relates to a method for treating pain in a patient, said method comprising the step of administering to the patient a pain-treating effective amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier, wherein the patient is suffering from enhanced or exaggerated sensitivity to pain, such as hyperalgesia, causalgia and allodynia; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional pain syndromes I and II; arthritic pain; sports injury pain; pain related to viral infection, *e.g.*, HIV, post-polio syndrome, and post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; post-chemotherapy pain; post-stroke pain; post-operative pain; physiological pain; inflammatory pain; acute inflammatory conditions/visceral pain, *e.g.,* angina, irritable bowel syndrome (IBS), and inflammatory bowel disease; neuropathic pain; neuralgia; painful diabetic neuropathy; traumatic nerve injury; spinal cord injury; and tolerance to narcotics or withdrawal from narcotics.

In an another preferred embodiment, the invention relates to a method for treating nerve tissue damage in a patient in need thereof, said method comprising the step of administering an effective nerve tissue damage-reducing amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier, wherein the patient is suffering from enhanced or exaggerated sensitivity to pain, such as hyperalgesia, causalgia and allodynia; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional pain syndromes I and II; arthritic pain; sports injury pain; pain related to viral infection, *e.g.*, HIV, post-polio syndrome, and post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; post-chemotherapy pain; post-stroke pain; post-operative pain; physiological pain; inflammatory pain; acute inflammatory conditions/visceral pain, *e.g.,* angina, irritable bowel syndrome (IBS), and inflammatory bowel disease; neuropathic pain; neuralgia; painful diabetic neuropathy; traumatic nerve injury; spinal cord injury; and tolerance to narcotics or withdrawal from narcotics.

Human MMP-2 and MMP-9 polypeptides may be employed in a process for screening for compounds that activate (called agonists) or inhibit the activation of (called antagonists) human MMP-2 and MMP-9 polypeptides.

Thus, human MMP-2 and MMP-9 polypeptides may also be used to assess the binding of small molecule substrates in, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. These substrates may be natural substrates or may be structural or functional mimetics. *See* Coligan, *et al., Current Protocols in Immunology* 1(2):Chapter 5 (1991).

Human MMP-2 and MMP-9 proteins are responsible for many biological functions, including many pathologies. Provided by the invention are screening methods to identify compounds that stimulate or that inhibit the function the function or level of the polypeptide. In general, agonists or antagonists are employed for diseases and disorders including, but not limited to: stroke; hemorrhage; reperfusion injury; cerebral ischemia; cerebral infarction; enhanced or exaggerated sensitivity to pain, such as hyperalgesia, causalgia and allodynia; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional

pain syndromes I and II; arthritic pain; sports injury pain; pain related to viral infection, *e.g.*, HIV, post-polio syndrome, and post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; post-chemotherapy pain; post-stroke pain; post-operative pain; physiological pain; inflammatory pain; acute inflammatory conditions/visceral pain, *e.g.*, angina, irritable bowel syndrome (IBS), and inflammatory bowel disease; neuropathic pain; neuralgia; painful diabetic neuropathy; traumatic nerve injury; spinal cord injury; and tolerance to narcotics or withdrawal from narcotics.

In general, such screening procedures involve providing appropriate cells that express human MMP-2 and/or MMP-9 polypeptide on the surface thereof. Such cells include cells from mammals, yeast, *Drosophila* or *E. coli*. In particular, a polynucleotide encoding human MMP-2 and/or MMP-9 is employed to transfect cells to thereby express human MMP-2 and/or MMP-9 polypeptide. The expressed polypeptide is then contacted with a test compound to observe binding, stimulation or inhibition of a functional response.

In a preferred embodiment of the invention, the screening protocol for dual MMP-2 and MMP-9 inhibitors set forth in Example 3 is employed.

An alternative screening procedure involves the use of melanophores that are transfected to express human MMP-2 polypeptide. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such an assay may be employed to screen for a compound that inhibits activation of human MMP-2 polypeptide by contacting the melanophore cells encoding the polypeptide (SEQ ID NO:4) with a compound to be screened. Inhibition of the signal generated by the ligand indicates that a compound is a potential antagonist for the polypeptide, *i.e.*, inhibits activation of the polypeptide. This same technique can be employed to find compounds that inhibit activation of MMP-9 polypeptide.

This technique may also be employed for screening of compounds that activate the polypeptide by contacting such cells with compounds to be screened and determining whether such a compound generates a signal, *i.e.*, activates the polypeptide(s).

Other screening techniques include the use of cells that express human MMP-2 and/or MMP-9 polypeptide(s) (for example, transfected CHO cells) in a system that measures extracellular pH changes caused by polypeptide activation. In this technique, compounds may be contacted with cells expressing the polypeptide. A second messenger response, *e.g.*, signal transduction or pH changes, is then measured to determine whether the potential compound activates or inhibits the polypeptide.

Another screening technique involves expressing the MMP-2 or MMP-9 polypeptides, such that they are linked to phospholipase C or D. Representative examples of such cells include, but are not limited to, endothelial cells, smooth muscle cells, and embryonic kidney cells. The screening may be accomplished as hereinabove described by detecting activation of the polypeptides or inhibition of activation of the polypeptides from the phospholipase second signal.

Another screening technique for antagonists or agonists involves introducing RNA encoding the human MMP-2 or MMP-9 polypeptide into *Xenopus* oocytes (or CHO, HEK 293, RBL-2H3, *etc.*) to transiently or stably express the polypeptide. The polypeptide oocytes are then contacted with a compound to be screened. Inhibition or activation of the polypeptide is then determined by detection of a signal, such as, cAMP, calcium, proton, or other ions.

Another method involves screening for human MMP-2 or MMP-9 polypeptide inhibitors by determining inhibition or stimulation of human MMP-2 or MMP-9 polypeptide-mediated cAMP and/or adenylate cyclase accumulation or dimunition. Such a method involves transiently or stably transfecting a eukaryotic cell with human MMP-2 or MMP-9 polypeptide to express the polypeptide on the cell surface. The cell is then exposed to potential antagonist compounds. The changes in levels of cAMP are then measured over a defined period of time, for example, by radio-immuno or protein binding assays (for example using Flashplates or a scintillation proximity assay). Changes in cAMP levels can also be determined by directly measuring the activity of the enzyme, adenylyl cyclase, in broken cell preparations. If the potential antagonist inhibits the activation of human MMP-2 or

MMP-9, the levels of MMP-2 or MMP-9 polypeptide-mediated cAMP, or adenylate cyclase activity, will be reduced or increased.

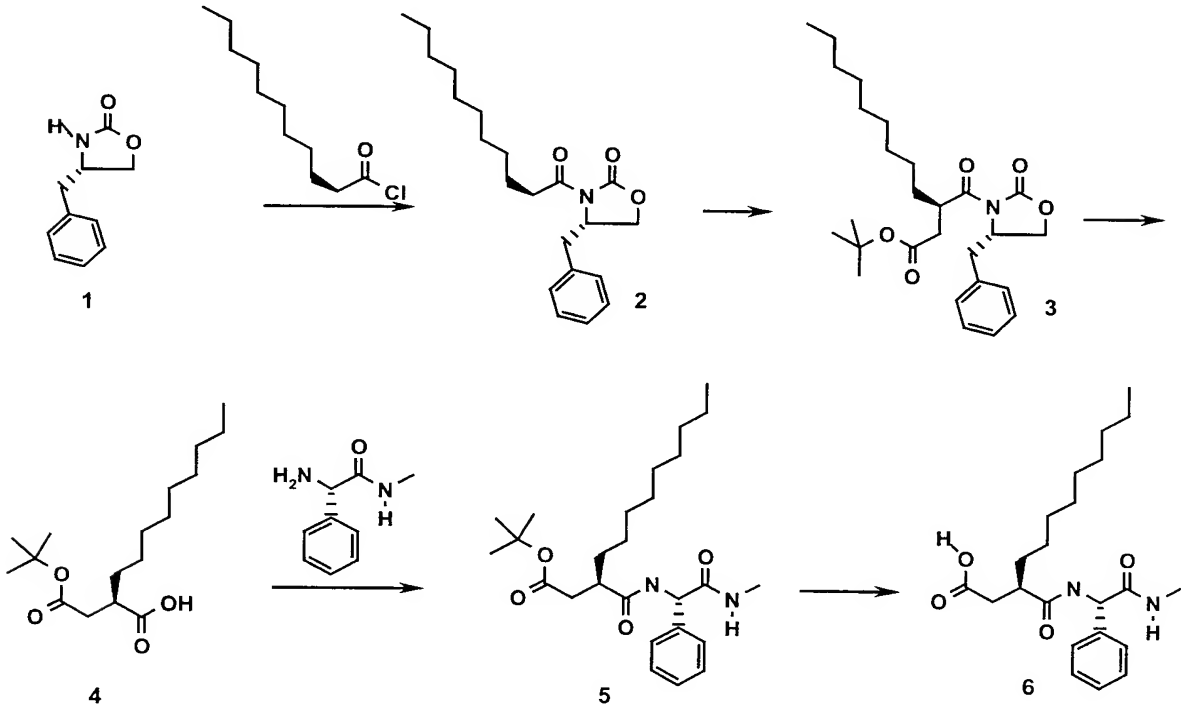
Another embodiment of the present invention relates to the agonists and antagonists obtainable from the above described screening methods.

Examples of potential human dual MMP-2 and MMP-9 polypeptide antagonists include peptidomimetics, synthetic organic molecules, natural products, antibodies, *etc.*, that bind to the polypeptide, but do not elicit a second messenger response, such that the activity of the polypeptide is prevented. Examples of small molecules include, but are not limited to, small peptides or peptide-like molecules.

Potential antagonists also include soluble forms of human MMP-2 and MMP-9 polypeptides, *e.g.*, fragments of the polypeptides.

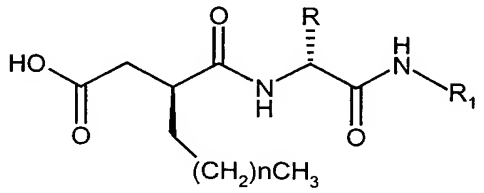
In a preferred embodiment of the present invention, the dual MMP-2 and MMP-9 antagonist compound administered to a patient in need thereof for treating pain is N-[2(R)-(n-Nonyl)succinyl]-L-phenylglycine-N-methylamide. This compound is prepared by methods analogous to that shown in Scheme 1.

Scheme I



The above-referenced antagonist was identified by the screening assay disclosed in Example 3. The method of synthesis of this dual antagonist is exemplified in Example 4.

In another particularly preferred embodiment, the dual MMP-2 and MMP-9 antagonist compounds administered to a patient in need thereof for treating pain caused by nerve tissue damage are selected from Formula (I), hereinbelow. Compounds of Formula (I) have the following structure:



Formula (I) wherein:

R is selected from a group consisting of alkyl, aryl, arylalkyl, heteroaryl, heteroalkylaryl,alkylthioalkyl, hydroxyalkyl, and aminoalkyl;

n represents an integer from seven to thirteen; and

R_1 is selected from the group consisting of alkyl, cycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl, and aminoalkyl wherein the amino may be unsubstituted, mono or disubstituted with an alkyl or aryl group or be part of a heterocyclic ring.

The aryl groups of R and R_1 may be substituted with groups such as alkyl, alkenyl, arylalkyl, acyl, aroyl, haloalkyl, halo, carboxy, carboalkoxy, carbamyl, alkylcarbamyl, arylcarbamyl, cyano, alkoxy, hydroxyl, phenylazo, amino, nitro, alkylamino, arylamino, arylalkylamino, acylamino, aroylamino, alkylthio, arylalkylthio, arylthio, alkysulfinyl, arylsulfinyl, arylalkylsulfinyl, alkylsulfonyl, arylsulfonyl, arylalkylsulfonyl, sulfamyl, arylsulfonamido, or alkylsulfonamido.

As used herein, "alkyl" refers to an optionally substituted hydrocarbon group joined together by single carbon-carbon bonds. The alkyl hydrocarbon group may be linear, branched or cyclic, saturated or unsaturated. Preferably, the group is unsubstituted. Preferably, the group is saturated. Preferred alkyl moieties are C1-5 alkyl.

As used herein, "aryl" refers to an optionally substituted aromatic group with

at least one ring having a conjugated pi-electron system, containing up to two conjugated or fused ring systems. "Aryl" includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. Preferred aryl moieties are phenyl, unsubstituted, monosubstituted, disubstituted or trisubstituted.

Preferred compounds, having formula (I), useful in the present invention are selected from the group consisting of:

N-[2(R)-Nonylsuccinic acid]-L-tyrosine-N-3-(N-morpholino)propylamide

N-[2(R)-Nonylsuccinic acid]-L-phenylglycine-N-3-(N-morpholino)propylamide

N-[2(R)-Nonylsuccinic acid]-L-leucine-N-3-(N-morpholino)propylamide

N-[2(R)-Nonylsuccinic acid]-L-methionine-N-3-(N-morpholino)propylamide

N-[2(R)-Nonylsuccinic acid]-L-tyrosine-N-2-(N-morpholino)ethylamide

N-[2(R)-Nonylsuccinic acid]-L-phenylalanine-N-3-(N-morpholino)propylamide

N-[2(R)-Nonylsuccinic acid]-L-valine-N-2-(N-morpholino)ethylamide

N-[2(R)-Nonylsuccinic acid]-L-tyrosine-N-(4-methoxyphenyl)amide

N-[2(R)-Nonylsuccinic acid]-L-phenylalanine-N-(4-methoxyphenyl)amide

N-[2(R)-Nonylsuccinic acid]-L-norvaline-N-(4-methoxyphenyl)amide

N-[2(R)-Nonylsuccinic acid]-L-arginine-N-(4-methoxyphenyl)amide

N-[2(R)-Nonylsuccinic acid]-L-phenylglycine-N-methylamide

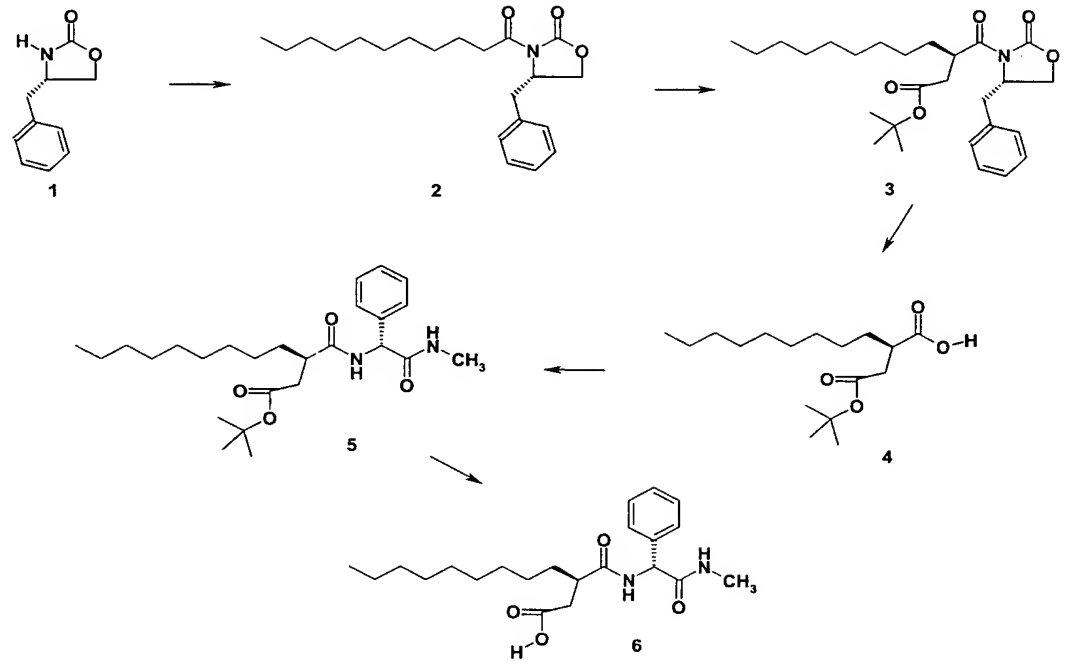
N-[2(R)-Nonylsuccinic acid]-L-tyrosine-N-cyclopentylamide

N-[2(R)-Nonylsuccinic acid]-L-tyrosine-N-3-dimethylaminopropylamide

Also included in this group of compounds are pharmaceutically acceptable salts and complexes of compounds of the Formula(I). Preferred are the zinc, copper, nickel, cobalt and rhodium complexes, hydrochloride, hydrobromide and trifluoroacetate salts. These compounds may contain one or more asymmetric carbon atoms and may exist in racemic and optically active forms. All of these compounds and diastereomers are contemplated to be within the scope of the present claimed screening methods.

In a preferred embodiment, the dual inhibitor employed in the claimed methods of treatment is N-[2(R)-nonylsuccinic acid]-L-phenylglycine-N-methylamide. This compound was prepared by methods analogous to that shown in Scheme 2.

Scheme 2

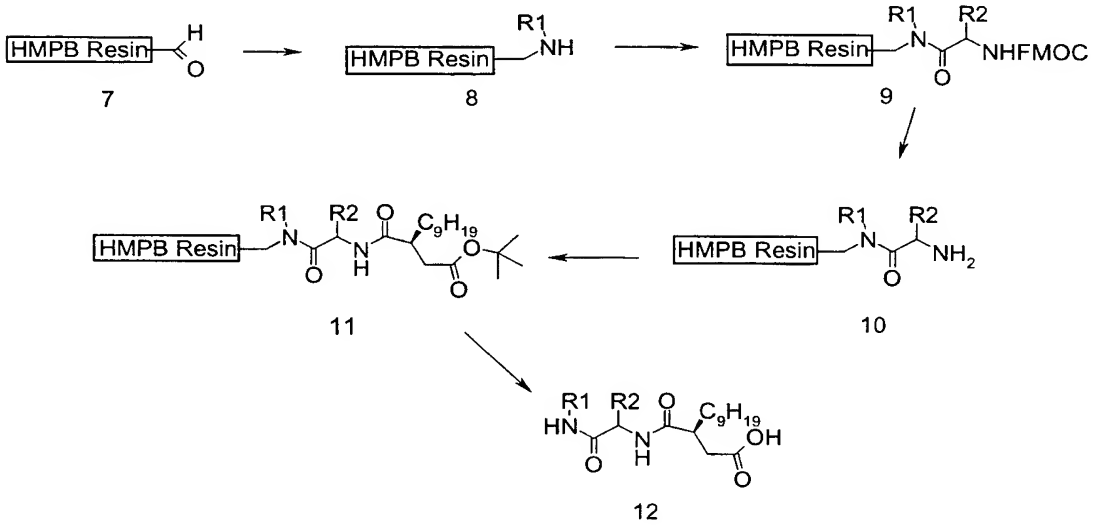


This compound was identified by the screening assay disclosed in Example 3, and its method of synthesis is exemplified in Example 5.

The compounds of this invention may also be prepared in an array format on polystyrene resin. To prepare N-[2(R)-Nonylsuccinic acid]-L-phenylalanine-N-3-(N-morpholino)propylamide N-(3-aminopropyl)morpholine was condensed with (4-Formyl-3,5-dimethoxyphenoxy)methyl polystyrene resin using sodium triacetoxyborohydride as the reducing agent. The product was coupled with (S)-Fmoc-phenylalanine using 1-hydroxy-7-azabenzotriazole (0.25 mmol) and di-isopropylcarbodiimide. The Fmoc proteacting group was removed with piperidine and the resulting product coupled with R-2- nonylsuccinic acid, 4-t-butyl ester using 1-hydroxy-7-azabenzotriazole and di-isopropylcarbodiimide. N-[2(R)-Nonylsuccinic acid]-L-phenylalanine-N-3-(N-morpholino)propylamide was obtained by treating the resin with trifluoroacetic acid and purification by automated preparative HPLC. LCMS analysis found that the product had the anticipated molecular weight of 517.

By a similar procedure, compounds were prepared using as amines methyl amine, 2-aminomethylpyridine, dimethylaminopropyl amine, 4-methoxyphenethyl amine, cyclopentyl amine, p-anisidine, 4-(3-aminopropyl)morpholine, and 2-aminoethylmorpholine and using as FMOC amino acids Fmoc-glycine, Fmoc-serine, Fmoc-valine, Fmoc-norvaline, Fmoc-leucine, Fmoc-isoleucine, Fmoc-phenylalanine, t-BuO-Fmmoc-tyrosine, Fmoc-methionine, Fmoc-D-homophenylalanine, Fmoc-phenyglycine, and Fmoc-lysine. Similar compounds may be prepared by using other amines for the reductive amination and other FMOC amino acids.

Scheme 3



With appropriate manipulation and protection of any chemical functionality, synthesis of the remaining compounds of Formula (I) is accomplished by methods analogous to those above and to those described in the Examples.

Prophylactic and Therapeutic Methods

This invention provides methods of treating an abnormal conditions related to both an excess of and insufficient amounts of human MMP-2 and MMP-9 activity.

If the activity of human MMP-2 and MMP-9 is in excess, several approaches are available. One approach comprises administering to a subject an inhibitor compound (antagonist) as hereinabove described, along with a pharmaceutically

acceptable carrier, in an amount effective to inhibit activation of MMP-2 and MMP-9, or by inhibiting a second signal, and thereby alleviating the abnormal condition.

In still another approach, expression of the gene encoding endogenous human MMP-2 and MMP-9 can be inhibited using expression blocking techniques. Known such techniques involve the use of antisense sequences, either internally generated or separately administered. See, for example, O'Connor, *J. Neurochem.* 56:560 (1991) in Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Alternatively, oligonucleotides that form triple helices with the gene can be supplied. See, for example, Lee, *et al., Nucleic Acids Res* 6:3073 (1979); Cooney, *et al., Science* 241:456 (1988); Dervan, *et al., Science* 251:1360 (1991). These oligomers can be administered per se or the relevant oligomers can be expressed *in vivo*.

For treating abnormal conditions related to an under-expression of human MMP-2 and MMP-9 and their activity, several approaches are also available. One approach comprises administering to a subject a therapeutically effective amount of a compound that activates human MMP-2 and MMP-9, *i.e.*, an agonist as described above, in combination with a pharmaceutically acceptable carrier, to thereby alleviate the abnormal condition. Alternatively, gene therapy may be employed to effect the endogenous production of human MMP-2 and MMP-9 by the relevant cells in the subject. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct may then be isolated and introduced into a packaging cell transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a subject for engineering cells *in vivo* and expression of the polypeptide *in vivo*. For overview of gene therapy, see Chapter 20, Gene Therapy and other Molecular Genetic-based Therapeutic Approaches, (and references cited therein) in HUMAN MOLECULAR GENETICS, T Strachan and A P Read, BIOS Scientific Publishers Ltd. (1996).

Formulation and Administration

Peptides, such as the soluble form of human MMP-2 and MMP-9 polypeptides, and agonists and antagonist peptides or small molecules, may be formulated in combination with a suitable pharmaceutical carrier. Such formulations comprise a therapeutically effective amount of the polypeptide or compound, and a pharmaceutically acceptable carrier or excipient. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. Formulation should suit the mode of administration, and is well within the skill of the art. The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compounds of the invention.

Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

Preferred forms of systemic administration of the pharmaceutical compositions include injection, typically by intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if properly formulated in enteric or encapsulated formulations, oral administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels, and the like.

The dosage range required depends on the choice of peptide, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, however, are in the range of 0.1-100 μg/kg of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

Polypeptides used in treatment can also be generated endogenously in the subject, in treatment modalities often referred to as "gene therapy" as described above. Thus, for example, cells from a subject may be engineered with a polynucleotide, such as a DNA or RNA, to encode a polypeptide ex vivo, and for example, by the use of a retroviral plasmid vector. The cells are then introduced into the subject.

BIOLOGICAL METHODS / EXAMPLES

Example 1: MMP-9 and MMP-2 Expression following Middle Cerebral Artery Occlusion in the Spontaneously Hypertensive Rat (Animal Model for Stroke)

Focal stroke was produced by permanent middle cerebral artery occlusion (MCAO) in the spontaneously hypertensive rat and MMP protein expression was measured by western blot and zymogram analysis over a time-course ranging from 6 hours to 30 days (n=32). Immunohistochemistry at 1 and 5 days (n=8 and 6, respectively) was also utilized to characterize the expression of several MMPs and related proteins following stroke, including their cellular source. To test the hypothesis that early increased MMP-9 expression is involved in ischemic brain injury, a neutralizing monoclonal antibody directed against MMP-9 was administered intravenously (n=7/group) 1 hour pre-MCAO and infarct size was measured 24 hours later.

a. Focal Brain Ischemia

Cerebral focal ischemia or sham surgery was carried out in male spontaneously hypertensive rats (SHR; Taconic Farms, Germantown, NY) at 16-18 weeks of age weighing 250-330 gm by permanent middle cerebral artery occlusion (MCAO) as described in detail previously (Clark, *et al.*, *Brain Res Bull* 31: 565-572 (1993); Barone, *et al.*, *Mol Chem Neuropath*; 24: 13-30 (1995); Barone, *et al.*, *Neurosci Biobehav Rev* 16: 219-233 (1992)). The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996. Briefly, the animals were anesthetized with pentobarbital (60 mg/kg, i.p.), and the middle cerebral artery (MCA) was occluded and cut dorsal to the lateral olfactory tract at the level of the inferior cerebral vein using electrocoagulation (Force 2 Electrosurgical Generator,

Valley Lab Inc., Boulder, CO). In sham-operated rats, the dura was opened over the MCA, but the artery was not occluded. Body temperature was maintained at 37°C until recovery from anesthesia. Rats were later euthanized with an overdose of pentobarbital, and the forebrains were removed for cortical dissection at various times following MCAO. In some cases, the ischemic frontoparietal cortex was dissected from the ipsilateral hemisphere, and the contralateral cortex was dissected as the non-ischemic control from the same rat (Barone, *et al.*, *Mol Chem Neuropath*; 24: 13-30 (1995)). Cortical samples were frozen immediately and stored at -80°C until used for protein analysis, as discussed below. Coronal sections were also made through the forebrain and the tissue was prepared for immunohistochemistry (Clark, *et al.*, *Brain Res Bull* 31: 565-572 (1993)). The time-points selected for observation post-stroke (6, 12, and 24 hours, and 5, 15, and 30 days) in the present study were based on earlier histologic characterization of this model in terms of ischemic injury, cellular infiltrate, and resolution of tissue damage by Clark, *et al.*, *Brain Res Bull* 31: 565-572 (1993). Ipsilateral cortical samples from sham-operated animals sacrificed at 12 and 24 hours and the contralateral cortex of all operated focal ischemic rats were compared. Several animals (n=4-8) were included at each time-point under each condition for comparison in each of the techniques that are described below.

b. Preparation of Tissue Extracts

To analyze protein expression patterns in control and ischemic brain tissue, protein extracts of the tissues were prepared. The forebrains were removed from each animal at various times after surgery and cortical samples were dissected as described above. Immediately after dissection, the tissues were stored at -80°C until all the samples from the time-course were collected. To prepare the tissues for extraction, they were first weighed and then minced into 1 mm^3 pieces. The minced tissues were incubated in an extraction buffer consisting of 0.5% Triton X-100 (Sigma) in PBS containing 0.5 U/ml aprotinin (Sigma) and 0.01% sodium azide while gently rotating at 4°C for 18 hours. The concentration of the initial extraction mixture for each tissue sample was normalized to 500 mg/ml. After the extraction was complete, the samples were centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatants were collected, aliquoted into 10 µl volumes and stored at -20°C.

To check the quality and uniformity of each extraction throughout the study (*i.e.*, to demonstrate that the extractions were consistent between time-points and animals evaluated), samples of each extract prepared were analyzed by SDS-PAGE (10% polyacrylamide) in which the gel was stained with 0.25% Coomassie Brilliant Blue 250 (Sigma).

c. Western Blot Analysis

To investigate the protein expression of various MMPs in control and ischemic tissue extracts, equal volumes (10 µl) of tissue extracts normalized for protein concentration, were prepared for Western analysis. Briefly, extracts were resolved by electrophoresis through a 10% polyacrylamide gel under reducing conditions (Laemmli, *Nature* 227: 680-6854 (1970)) and then transferred to a nitrocellulose membrane. Unoccupied binding sites were blocked overnight at 4°C with 5% nonfat powdered milk in a 0.1 M Tris-HCl buffer, pH 8.0, containing 1.5 M NaCl and 0.5% Triton X-100 (TBST buffer). A primary antibody, diluted in TBST, was then added to the membrane and allowed to incubate for 1 hour at 25°C. The membrane was washed three times, 15 minutes each, with TBST and then incubated for 30 minutes with a secondary antibody conjugated to horseradish peroxidase (Sigma). The membrane was washed as above and the blot was developed using the enhanced chemiluminescence method (Amersham) according to the manufacturer's instructions. The primary antibodies used included the following: mouse monoclonal antibodies directed against MMP-1 (clone# 41-IE5, Oncogene Science); MMP-2 (clone# 42-5D11, Oncogene Science); and MMP-3 (clone# 55-2A4, Oncogene Science); and rabbit polyclonal antibodies directed against MMP-3 (Biogenesis); and MMP-9, a gift from Dr. Hideaki Nagase, University of Kansas Medical Center.

d. SDS-PAGE Zymography

MMP enzyme expression was assayed by zymography, as described by Herron, *et al.*, *J Biol Chem* 261: 2814-2818 (1986). Briefly, equal volumes (10 µl) of tissue extracts normalized for protein concentration, were subjected to electrophoresis, without boiling or reduction, through a 10% polyacrylamide gel co-polymerized with gelatin (0.5 mg/ml) or casein (0.5 mg/ml) at 4°C. After

electrophoresis was complete, the gel was incubated for 1 hour at 25°C in a 2.5% Triton X-100 solution, washed two times, 20 minutes each, with water and then incubated overnight at 37°C in a 0.05 M Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$. As a control, duplicate samples were loaded onto another gel, which was then incubated in a 0.05 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA to inhibit MMP activity. The gels were fixed with 40% methanol and 7% acetic acid, stained with 0.25% Coomassie Blue R250 and then destained with 10% methanol and 7% acetic acid. Enzyme activity attributed to MMP-1, MMP-2 and MMP-9 was visualized (on the basis of molecular weight) in the gelatin-containing zymograms as clear bands against a blue background. Similarly, casein-containing zymograms were used to determine MMP-3 activity. To quantitate the relative levels of MMP expression detected by SDS-PAGE zymography, the gels were digitized and the area of lysis for each band detected was quantitated by computer-assisted planimetry of the lytic zone area in mm^2 (Amersham RAS 3000 Image Analysis System; Loats Associates, Inc.).

e. *In Situ* Zymography

Although SDS-PAGE zymograms are useful in identifying the presence of latent ("pro") and active forms of various MMPs, they cannot indicate the actual net proteolytic activity due to the presence of tissue inhibitors of metalloproteinases (TIMPs). TIMPs are generally coexpressed with MMPs in tissue samples and can inhibit MMP activity. However, the presence of SDS in SDS-PAGE zymograms displaces TIMPs from the MMPs and also activates latent enzymes. Therefore, to analyze net endogenously active MMP expression within the brain tissue following MCAO, *in situ* zymography was conducted. With this method, endogenous MMP activity could then be identified and correlated to a particular region of the tissue. Brain tissue from animals that had undergone focal cerebral ischemic or sham surgery were removed after 24 hours and immediately placed on ice. Coronal sections (1 mm) were made through the forebrain and rinsed briefly in PBS. The sections were then directly overlaid on top of a gel consisting of 10% polyacrylamide copolymerized with gelatin (0.5 mg/ml) in 50 mM Tris, 5 mM $CaCl_2$, pH 7.4. The tissue sections were incubated on top of the gel for 8 hours at

37°C, after which the sections were removed and the gel was incubated further for a total of 18 hours. The gels were fixed with 40% methanol and 7% acetic acid, stained with 0.25% Coomassie Blue R250 and then destained with 10% methanol and 7% acetic acid. Gelatinase activity was visualized as a zone of clearing against a blue background.

f. Immunohistochemistry

Forebrains were removed from animals that had undergone permanent focal cerebral ischemia after 24 hours (n=8) or 5 days (n=6) and immediately placed on ice. Sham operated rats sacrificed after 24 hours (n=5) or 5 days (n=5) were utilized as controls. Coronal sections (2 mm) were made through the forebrain, after which the sections were fixed with 10% (wt/vol) phosphate-buffered formalin (Baxter Scientific Products) for 18-20 hours. Following standard histological processing and embedding in paraffin, 6 μm-thick sections were prepared for immunoperoxidase staining using the Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer's instructions. Briefly, endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol for 30 minutes. Nonspecific immunoglobulin binding sites were blocked with normal goat serum for 1 hour, and then the sections were incubated with a primary monoclonal antibody for 1 hour at room temperature. As a negative control, serial sections were incubated with mouse IgG (Vector Laboratories) instead of the primary antibody. The sections were then incubated for 30 minutes with a biotinylated goat anti-mouse IgG secondary antibody (1:200, Vector Laboratories) followed by 30 minutes of incubation with the Vectastain Elite ABC reagent solution. Immunoglobulin complexes were visualized upon incubation with 3,3'-diaminobenzidine (DAB, Vector Laboratories) at 0.5 mg/ml in 50 mM Tris-HCl, pH 7.4 and 3% H_2O_2. DAB staining was enhanced by treating the sections for 10 seconds with DAB Enhancing Solution (Vector Laboratories). Sections were washed, counterstained with Gill's Hematoxylin, cleared, mounted with Aquamount (Polysciences), and then examined by light microscopy using an Olympus IX70 microscope. The primary monoclonal antibodies used for these studies were: anti-MMP-1 (clone# 41-IE5, Oncogene Science); anti-MMP-2 (clone# 42-5D11, Oncogene Science); anti-MMP-3 (clone# 55-2A4, Oncogene Science);

anti-MMP-9 (clone# 56-2A4, Oncogene Science), anti-TIMP-1 (clone# 7-6C1, Oncogene Science), anti-neurofilament-200 (N52, Sigma), and an antibody that recognizes monocytes and macrophages (clone ED1, BioSource).

g. Statistical Analysis

Data were expressed as mean ± SEM. For statistical analysis of gel lytic zone data, an Analysis of Variance was followed up with the Fisher's Least Significant Difference Test. Statistical significance was accepted when $p<0.05$.

h. Results

MMP expression increased progressively over time following stroke. After 12 hours, significant ($p<0.05$) MMP-9 activity was observed that reached maximum levels by 24 hours ($p<0.001$), then persisted for 5 days at this level and returned to basal (zero) levels by 15 days. Based on morphologic criteria, MMP-9 appeared to stain with endothelial cells and neutrophils identified both within and at the periphery of the infarct within 24 hours of focal ischemia. After 5 days, MMP-9 appeared to stain with macrophages present within the infarcted brain. MMP-2 activity was significantly ($p<0.001$) increased by 24 hours and was maximum after 5 days post-MCAO. MMP-2 appeared to stain with macrophages present within the infarcted region. Unlike MMP-9 and MMP-2, tissue inhibitor of metalloproteinase-1 (TIMP-1) was identified at comparable levels in both control and ischemic tissue following MCAO. MMP-1 and MMP-3 could not be detected in the brain following focal stroke. When an MMP-9 neutralizing monoclonal antibody was administered systemically, animals exhibited significantly reduced infarct size (*i.e.*, a 30% reduction compared to non-immune antibody controls, $p<0.05$).

These results demonstrate that early increased MMP-9 expression in endothelial cells and infiltrating neutrophils is a significant response to cerebral focal ischemia, and that selective inhibition of MMP-9 activity can significantly reduce brain injury following stroke. The expression of MMP-2 also participates in tissue damage, but early expression may contribute to wound healing. These results demonstrate that a dual MMP-9/MMP-2 inhibitor would be of benefit for patients suffering from stroke, hemorrhage, reperfusion injury, cerebral ischemia, and cerebral infarction.

Example 2: MMP-9 and MMP-2 Expression following Sciatic Nerve Injury

a. Preparation of Tissue Extracts

To analyze protein expression patterns in control and injured sciatic nerve tissue, protein extracts of the tissues were prepared. The sciatic nerves were dissected from each animal and then minced into 1 mm^3 pieces. The minced tissues were incubated in an extraction buffer consisting of 0.5% Triton X-100 (Sigma, St. Louis, MO) in PBS containing 0.5 U/ml aprotinin (Sigma, St. Louis, MO) and 0.01% sodium azide while gently rotating at 4°C for 18 hours. After the extraction was complete, the samples were centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatants were collected, aliquoted into 10 µl volumes and stored at -20°C. To check the quality and uniformity of each extraction throughout the study (*i.e.*, to demonstrate that the extractions were consistent between time-points and animals evaluated), samples of each extract prepared were analyzed by SDS-PAGE (10% polyacrylamide) in which the gel was stained with 0.25% Coomassie Brilliant Blue 250 (Sigma, St. Louis, MO).

b. SDS-PAGE Zymography

MMP enzyme expression was assayed by SDS-PAGE zymography using gelatin as an MMP substrate. For gelatin-containing zymograms, equal volumes (10 µl) of tissue extracts normalized for protein concentration were subjected to electrophoresis, without boiling or reduction, through a 10% polyacrylamide gel co-polymerized with gelatin (0.5 mg/ml) at 4°C. After electrophoresis was complete, the gel was incubated for 1 hour at 25°C in a 2.5% Triton X-100 solution, washed two times, 20 minutes each, with water and then incubated overnight at 37°C in a 0.05 M Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$. As a control, duplicate samples were loaded onto another gel that was then incubated in a 0.05 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA to inhibit MMP activity. The gels were fixed with 40% methanol and 7% acetic acid, stained with 0.25% Coomassie brilliant blue R250 and then destained with 10% methanol and 7% acetic acid. Enzyme activity attributed to MMP-1, MMP-2 and MMP-9 were visualized in the gelatin-containing zymograms as clear bands against a blue background. Standards

for the active forms of MMP-2 and MMP-9 were included on the gels for comparison and identification.

c. Western Blot Analysis

To investigate the protein expression of various MMPs in control and injured tissue extracts, equal volumes (10 µl) of tissue extracts normalized for protein concentration, were prepared for Western analysis. Briefly, extracts were resolved by electrophoresis through a 12% polyacrylamide gel under reducing conditions and then transferred to a nitrocellulose membrane. Unoccupied binding sites were blocked overnight at 4°C with 5% nonfat powdered milk in a 0.1 M Tris-HCl buffer, pH 8.0, containing 1.5 M NaCl and 0.5% Triton X-100 (TBST buffer). A polyclonal primary antibody, diluted in TBST, was then added to the membrane and allowed to incubate for 1 hour at 25°C. The membrane was washed three times, 15 minutes each, with TBST and then incubated for 30 minutes with a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Bio-Rad, Richmond, CA). The membrane was washed as above and the blot was developed using the enhanced chemiluminescence method (Amersham, Piscataway, NJ) according to the manufacturer's instructions. The primary antibodies used included the following: rabbit anti-TIMP-1 (5 µg/ml, Biogenesis, Sandown, NH) and rabbit anti-TIMP-2 (5 µg/ml, Biogenesis, Sandown, NH).

d. Summary of Results:

Rat sciatic nerves (n=3) were injured by chronic constriction injury (CCI). The tissues were then evaluated for matrix metalloproteinase (MMP) expression by gelatinase zymography. Western blot analysis was also conducted for TIMP-1 and TIMP-2 expression. TIMPs (tissue inhibitors of matrix metalloproteinases) are the endogenous inhibitors for MMPs. TIMP-1 is the preferred inhibitor for MMP-9, and TIMP-2 is the preferred inhibitor for MMP-9.

The results demonstrate that the control (sham operated) and contralateral control samples have low levels of MMP-9 and MMP-2. However, the injured samples have significant upregulation of both MMP-9 and MMP-2. Notably, these enzymes are in the active forms. Western blot results indicated that TIMP-1

expression is constitutive in all the samples, whereas a modest reduction in TIMP-2 expression was observed only in the injured samples.

These results indicate that MMPs, in particular MMP-9 and MMP-2, contribute to the tissue damage and inflammation that occurs following nerve injury. These events contribute to both acute and chronic pain experienced with injury. The results in this experiment demonstrate that inhibition of MMP-9 and/or MMP-2 expression and/or activity can significantly reduce nerve tissue damage and pain following injury.

Example 3: MMP-2/MMP-9 Screening Assay Protocol

A high-throughput, 96-well screen was used to measure MMP-9 activity and to detect potential inhibitors of MMP-9. The screen is a quenched fluorescence assay. The components of the assay include purified recombinant human MMP-9 (generated by SB, 3 nM final concentration) and a fluorogenic peptide substrate (Peptides International, Louisville, KY, 10 μM final concentration) incubated in the presence or absence of compound. Briefly, enzyme activity is measured after 30 minutes incubation at 37°C. and quantitated using a peptide substrate, (Dnp-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(NMa)-NH_2 or (2,4-Dinitrophenyl-L-Prolyl-L-Cyclohexylalanyl-Glycyl δ-Methyl-L-Cysteinyl-L-Histidyl-L-Alanyl-N^{ε}-Methylenthranoyl-L-Lysine Amide) containing a fluorophore, Nma, on one end of the peptide and a quencher, Dnp, on the other end. When the peptide is intact, the fluorophore is quenched. When the peptide is cleaved by MMP-9, the quencher is dissociated from the fluorophore and a fluorescent signal is emitted that can easily be detected using a fluorescent plate reader. The universal cleavage site within the peptide that is recognized by MMP-1, -2, -3, -9 and -13 is the Gly-Cys bond.

Compounds that exhibit an IC_{50} for MMP-9 that is less than 1 μm were subjected to additional screens using purified recombinant human MMP-2 (generated by SB, 10 nM), MMP-13 (Chemicon, Temecula, CA), MMP-3 (Biogenesis, Sandown, NH) and MMP-1 (Biogenesis, Sandown, NH) to confirm selectivity for MMP-9. These screens were conducted using the same fluorogenic peptide substrate as that described above for MMP-9. Using this screening protocol,

N-[2(R)-(n-Nonyl)succinyl]-L-phenylglycine-N-methylamide, was identified as a dual MMP-2/MMP-9 inhibitor. The synthesis of this compound is described in detail in Example 3.

Synthetic Chemistry / Examples

Example 4: Synthesis of N-[2(R)-(n-Nonyl)succinyl]-L-phenylglycine-N-methylamide

4(S)Benzyl-2-oxazolidine (21.6 g, 122 mmol)(1) dissolved in 250 ml of THF was cooled to –78° C and 61 ml (128 mmol) of 2.1 M butyl lithium in hexane added dropwise while keeping the temperature below –65° C. The mixture was kept chilled and stirred for 45 min, and a solution of 27.5 g (134 mmol) of undecanoyl chloride dissolved in 50 ml of THF added dropwise. The mixture was stirred at -78° for 1 hr, and then allowed to stir at ambient temperature for 18 hr. Then 20 ml of 1N HCl added and the mixture extracted with ethyl acetate. The organic layer was washed with water, saturated sodium bicarbonate, dried over $MgSO_4$ and concentrated under vacuum to give 4(S)-benzyl-3-undecanoyl-2-oxazolidinone (2) as an oil which was used without further purification.

A solution of 13.13 g (130 mmol) of diisopropylamine in 200 ml of THF was cooled to –78° C and a solution of 2.1 M butyl lithium in hexane (58 ml, 122 mmol) added dropwise. After 30 min at –78° C a solution of 2 (crude sample from above) in 150 ml of THF was added and the stirring continued at –78° C for 1 hr. Then t-butyl bromoacetate (26.5 g, 136 mmol) added and the mixture stirred for 18 hr at ambient temperature. To this was added 100 ml of 0.5 N HCl and the mixture extracted with EtOAc. The organic layer was washed with water, saturated saline, and dried over $MgSO_4$ to give 4(S)-benzyl-3-[2-(R)-[(tert-butoxycarbonyl)methyl]undecanoyl]-2-oxazolidinone (3) which was purified by chromatography on silica eluting with 10% ethyl acetate – hexane.

A solution of 10 g (21.7 mmol) of 3 in a mixture of 240 ml of THF and 72 ml of water was cooled to 0° C and added to a solution of 11 ml of 30% hydrogen peroxide and 1.56 g (37.2 mmol) of LiOH hydrate in 70 ml of water held at < 5° C. The solution was stirred for 2 h at ambient temperature, 6.5 g of $NaNO_2$ added and the solution

stirred an additional hour. The mixture was concentrated under vacuum to remove the THF, diluted with 5% Na_2CO_3, and extracted twice with ether. The pH of the aqueous solution was brought to 2 with conc HCl and extracted with EtOAc twice. The organic layer was dried and concentrated to give 2-(R)-[(tert-butoxycarbonyl)methyl]undecanoic acid (4).

A solution of L-phenylglycine-N-methylamide (3.1 g, 18.3 mmol), HOBT (2.47 g, 18.3 mmol), and 5.5g (18.3 mmol) of 4 in 60 ml of DMF was treated with 3.5 g, (18.3 mmol) of EDC hydrochloride and stirred at ambient temperature for 24 h. The reaction mixture was diluted with EtOAc and washed with 0.5 N HCl, water, saturated $NaHCO_3$, and saturated saline. It was then dried over $MgSO_4$, concentrated under vacuum, and chromatographed over silica eluting with 30% EtOAc – hexane to give 2-(R)-[(tert-butoxycarbonyl)methyl]undecanoyl-L-phenylglycine-N-methylamide (5).

A solution of 6.6 g (14.8 mmol) of 5 in 50 ml of 90% TFA was stirred for 2 h at ambient temperature. The solution was concentrated under vacuum, the residue treated with water, and then extracted with ethyl acetate. The ethyl acetate was dried, concentrated under vacuum, and the residue triturated with acetonitrile to give colorless crystals of N-[2(R)-(n-Nonyl)succinyl]-L-phenylglycine-N-methylamide (6), mp 156 – 158° C.

Example 5: Synthesis N-[2(R)-nonylsuccinic acid]-L-phenylglycine-N-methylamide

A synthesis of N-[2(R)-nonylsuccinic acid]-L-phenylglycine-N-methylamide was carried out as shown in Scheme 2 by reacting 4(S)Benzyl-2-oxazolidine with butyl lithium to give the nitrogen anion and then reaction of this with undecanoyl chloride to give (S)-4-benzyl-3-undecanoyl-oxazolidin-2-one (2). This was converted to the anion by reaction with lithium diisopropylamide and quenched with t-butyl bromoacetate to give 4(S)-benzyl-3-[2-(R)-[(tert-butoxycarbonyl)methyl]undecanoyl]-2-oxazolidinone (3) which was purified by chromatography. This product was converted to 2-(R)-[(tert-butoxycarbonyl) methyl]undecanoic acid (4) by hydrolysis with lithium hydroxide in the presence of hydrogen peroxide. L-Phenylglycine-N-methylamide was prepared by reaction of the methyl ester of L-phenylglycine with methyl amine, and this condensed with 4 in a

standard amide forming reaction to give 2-(R)-[(tert-butoxycarbonyl)methyl] undecanoyl-L-phenylglycine-N-methylamide (5). After purification by chromatography this was hydrolyzed by treatment with 90% trifluoroacetic acid to give the desired N-[2(R)-nonylsuccinic acid]-L-phenylglycine-N-methylamide which was crystallized from acetonitrile.

Example 6: N-[2(R)-Nonylsuccinic acid]-L-phenylalanine-N-3-(N-morpholino)propylamide prepared on polystyrene resin

Reductive amination of resin. (4-Formyl-3,5-dimethoxyphenoxy)methyl polystyrene resin (Polymer Laboratories, 1.82 mmol/gm., 10g) was suspended in a mixture of N-methylpyrrolidinone(100 mL) and acetic acid(25 mL) in a large shaker vessel. N-3-aminopropylmorpholine (0.1 mol) was added and the mixture shaken at room temperature for 1 h. Sodium triacetoxyborohydride(0.05 mol) was then added in N-methylpyrrolidine(50 mL) and the mixture was allowed to shake at room temperature overnight. The resin was then filtered, washed with 1:1 DMF/water(3 X), DMF(3 X), and dichloromethane(4 X).

Coupling of Fmoc-amino acid

The reductively aminated resin (50 mg) was suspended in 1 mL of N-methylpyrrolidinone. To this was added (S)-Fmoc-phenylalanine (0.25 mmol) , 1-hydroxy-7-azabenzotriazole (0.25 mmol) and di-isopropylcarbodiimide (0.25 mmol). The reaction mixture was shaken at room temperature overnight, filtered, washed with DMF(4 X) and the coupling repeated once more. The resin was filtered, washed with DMF(4 X) and dichloromethane(4 X).

Removal of FMOC group.

The product from the above step was treated with 20% piperidine in DMF (1.5 mL) and agitated for one hour. The resin was washed with DMF(4 X).

Coupling of R-2- nonylsuccinic acid, 4-t-butyl ester.

The product from the above step was suspended in N-methylpyrrolidinone(1 mL). To this was added R-2- nonylsuccinic acid, 4-t-butyl ester (0.25 mmol), 1-hydroxy-7-azabenzotriazole (0.25 mmol) and di-isopropylcarbodiimide (0.25 mmol).

The reaction mixture was shaken at room temperature for 16 h, filtered, and the resin washed with DMF(4 X), Methanol(4 X) and dicloromethane(4 X).
TFA cleavage to yield N-[2(R)-Nonylsuccinic acid]-L-phenylalanine-N-3-(N-morpholino)propylamide (12).

The resin obtained from the previous step was treated with trifluoroacetic acid(1.5 mL) and agitated for 8 h, filtered, and washed with dichloromethane. The combined filtrate was concentrated and subjected to purification using automated preparative HPLC and concentrated in a vacuum centrifuge The residue when analyzed by LCMS gave a molecular weight of 517.

The above description fully discloses the invention, including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. Therefore, the examples provided herein are to be construed as merely illustrative and are not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.

What is claimed is:

1. A method for treating pain in a patient in need thereof, said method comprising the step of administering an effective pain-reducing amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier.

2. The method as claimed in Claim 1, wherein the patient is suffering from a disease or disorder selected from the group consisting of: enhanced or exaggerated sensitivity to pain; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional pain syndromes I and II; arthritic pain; sports injury pain; pain related to viral infection; post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; post-chemotherapy pain; post-stroke pain; post-operative pain; physiological pain; inflammatory pain; acute inflammatory conditions/visceral pain; neuropathic pain; neuralgia; painful diabetic neuropathy; traumatic nerve injury; spinal cord injury; and tolerance to narcotics or withdrawal from narcotics.

3. A method for treating nerve tissue damage in a patient in need thereof, said method comprising the step of administering an effective nerve tissue damage-reducing amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier.

4. The method as claimed in Claim 3, wherein the patient is suffering from a disease or disorder selected from the group consisting of: stroke; hemorrhage; reperfusion injury; cerebral ischemia; cerebral infarction; enhanced or exaggerated sensitivity to pain; acute pain; burn pain; atypical facial pain; neuropathic pain; back pain; complex regional pain syndromes I and II; arthritic pain; sports injury pain; pain related to viral infection; post-herpetic neuralgia; phantom limb pain; labor pain; cancer pain; post-chemotherapy pain; post-stroke pain; post-operative pain; physiological pain; inflammatory pain; acute inflammatory conditions/visceral pain;

neuropathic pain; neuralgia; painful diabetic neuropathy; traumatic nerve injury; and tolerance to narcotics or withdrawal from narcotics.

5. A method for treating a patient suffering from a disease selected from the group consisting of: stroke, hemorrhage, reperfusion injury, cerebral ischemia,, and cerebral infarction, said method comprising the step of administering an effective amount of a dual inhibitor of human MMP-2 (SEQ ID NO:2) and MMP-9 (SEQ ID NO:4) in combination with a carrier.

Figure 1

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 151  cctgtttgtg ctgaaggaca cactaaagaa gatgcagaag ttctttggac
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 401  gtctggagcg atgtgacccc actgcggttt tctcgaatcc atgatggaga
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2151 tccccctcca atcccaccaa ccctcagagc cacccctaaa gagatacttt
2201 gatattttca acgcagccct gctttgggct gccctggtgc tgccacactt
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2401 cactgctggc tgccttagaa cctttcttac attagcagtt tgctttgtat
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2551 tctcagccca catagtgatg gttcccctgt tcactctact tagcatgtcc
2601 ctaccgagtc tcttctccac tggatggagg aaaaccaagc cgtggcttcc
2651 cgctcagccc tccctgcccc tcccttcaac cattccccat gggaaatgtc
2701 aacaagtatg aataaagaca cctactgagt ggc

Figure 2

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 61 MQKFFGLPQT GDLDQNTIET MRKPRCGNPD VANYNFFPRK PKWDKNQITY RIIGYTPDLD
121 PETVDDAFAR AFQVWSDVTP LRFSRIHDGE ADIMINFGRW EHGDGYPFDG KDGLLAHAFA
181 PGTGVGGDSH FDDDELWTLG EGQVVRVKYG NADGEYCKFP FLFNGKEYNS CTDTGRSDGF
241 LWCSTTYNFE KDGKYGFCPH EALFTMGGNA EGQPCKFPFR FQGTSYDSCT TEGRTDGYRW
301 CGTTEDYDRD KKYGFCPETA MSTVGGNSEG APCVFPFTFL GNKYESCTSA GRSDGKMWCA
361 TTANYDDDRK WGFCPDQGYS LFLVAAHEFG HAMGLEHSQD PGALMAPIYT YTKNFRLSQD
421 DIKGIQELYG ASPDIDLGTG PTPTLGPVTP EICKQDIVFD GIAQIRGEIF FFKDRFIWRT
481 VTPRDKPMGP LLVATFWPEL PEKIDAVYEA PQEEKAVFFA GNEYWIYSAS TLERGYPKPL
541 TSLGLPPDVQ RVDAAFNWSK NKKTYIFAGD KFWRYNEVKK KMDPGFPKLI ADAWNAIPDN
601 LDAVVDLQGG GHSYFFKGAY YLKLENQSLK SVKFGSIKSD WLGC
```

Figure 3

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Figure 4

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SEQUENCE LISTING

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BARONE, FRANK
BINGHAM, SHARON

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27949

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 2, 21, 564; 435/70.1, 219, 226, 325; 536/23.1, 23.2, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 6,114,159 A (WILL et al.) 05 September 2000, abstract, col. 1, lines 5-14, Table 1 and SEQ ID NOS:18 and 19.	1-5
Y	WO 98/39024 A1 (DARWIN DISCOVERY LIMITED) 11 September 1998, abstract, page 1, lines 17 to page 2, lines 22, page 3, lines 13 to page 4, lines 7 and page 9, lines 19 to page 20, lines 15.	1-5
Y	US 4,923,818 A (GOLDBERG et al.) 08 May 1990, col. 1, lines 66-68, Figures 3A to 3C and claims 1-2.	1-5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 JANUARY 2001	Date of mailing of the international search report 07 FEB 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorothea Lawrence for ABDEL A. MOHAMED Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27949

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COLLIER et al. On the Structure and Chromosome Location of the 72-and 92-kDa Human Type IV Collagenase Genes. Genomics. 1991, Vol. 9, pages 429-434, especially 429 and 432-433.	1-5
A	LEES et al. Mast cell proteinases activate precursor forms of collagenase and stromelysin, but not of gelatinase A and B. Eur. J. Biochem. 1994, Vol. 223, pages 171-177, especially pages 171 and 175-176.	1-5
A	TAMURA et al. Nitric Oxide Mediates Interleukin-1-Induced Matrix Degradation and Basic Fibroblast Growth Factor Release in Cultured Rabbit Articular Chondrocytes: A Possible Mechanism of Pathological Neovascularization in Arthritis. Endocrinolgy. 1996, Vol. 137, No. 9, pages 3729-3737, especially pages 3729 and 3734-3736.	1-5
A	MAKOWSKI et al. Identification and Partial Characterization of Three Calcium-and Zinc-Independent Gelatinases Constitutively Present in Human Circulation. Biochemistry and Molecular Biology International. December 1998, Vol. 46, No. 5, pages 1043-1053, especially pages 1043-1045, 1049 and 1052.	1-5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27949

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/12, 2, 21, 564; 435/70.1, 219, 226, 325; 536/23.1, 23.2, 23.5

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS,CAS ONLINE, DIALOG, MEDLINE, EMBASE, WPIDS
search terms: matrix metalloproteinase or MMP or MMP-2 or gelatinase A or MMP-9 or gelatinase B; treat? or therapeut? or adminster?; pain, neve tissue damage; diseas? or disorder?